METHOD FOR STIMULATING WOUND HEALING

This application claims the priority of Korea Patent Application No. 2002-42858, filed July 22, 2002.

FIELD OF THE INVENTION

The present invention relates to a method for stimulating wound healing, and more particularly to a method for stimulating wound healing in a subject in need thereof, comprising: administering to a wound of the subject an effective amount for stimulating wound healing of a composition, wherein the composition comprising p43 having an amino acid sequence set forth in SEQ ID NO: 1 or functional equivalents thereof.

BACKGROUND OF THE INVENTION

In general, wound healing is any reaction of tissue against damage inflicted upon the tissue, and it is known as a complex biological process comprising chemotaxis, cell differentiation and replication, synthesis of matrix protein, angiogenesis and reconstitution of wound, as a series of tissue repair processes (*Steed, D. L. et al., Clin. Plast. Surg. 25:397, 1998*). As one of the typical factors for controlling wound healing process, the growth factor may be considered, which regulate cell growth, cell differentiation, cell metabolism and the like through the general wound healing process, as well as the environment around a wound, and have consistently been used for the development of treatment agents. In addition, various kinds of cytokine are known to participate in wound healing. As an

example of the cytokine, there is transforming growth factor-β (TGF-β). The TGF-β is involved in the growth and differentiation of various cells. It has been reported that the TGF-β is involved in several complicated functions including growth control, the regulation of immune response, the stimulation of osteogenesis, the induction of cartilage-specific macromolecule and the promotion of wound healing (Bennett, N. T. *et al.*, *Am. J. Surg.* 165:728, 1993).

According to whether skin deficiency exists or not, a wound may be classified into two types and may be treated with different healing mechanisms. When a wound does not involve skin deficiency, as in most mild injuries, for example a case of having injury only to the epidermis, keratinocytes move from the wound periphery, cover the wound, and reform new epidermis and keratin (Knighton, D. R. and Fiegel, V. D., *Invest. Radiol.* 26:604-611, 1991). However, when entire skin layers are totally injured or destroyed, new connective tissues, called granulation tissues, fill wound space where regenerated epidermis from the periphery of the wound sites must be covered. The granulation tissues are formed by the deposition of extracellular matrix components, such as collagen, from fibroblasts moving into the wound space. Consequently, the healing mechanisms of wound significantly varied depending on the presence of such skin deficiency. Successful wound healing requires completing a series of wound healing multistage processes. Since the deletion of one or more of wound healing-related components prevents the wound healing and skin repair, the wound remains exposed. Such an exposed wound can be easily infected, resulting in delayed healing processes, and the formation of ulcers and erosion of skin. There is a need for the development of a drug promoting the growth of granulation tissue and the regeneration of skin on wound involving skin deficiency.

Meanwhile, p43 is a protein consisting of 312 amino acids which is associated with a multi-tRNA synthetase complex to facilitate the catalytic activity of the bound enzyme. The p43 is highly expressed by microneuron in the lesions of autoimmune diseases in vitro including encephalomyelitis, neuritis and uveitis. The phenomenon in which p43 is highly expressed at certain developmental stages and tissues suggests that p43 is related to inflammation response and cell apoptosis (Berger, A. C. *et al.*, *J. Immunother.* 23:519-527, 2000). We have previously shown that p43 can be employed as an effective cytokine and an anti-tumor agent (PCT International application No. PCT/KR00/00630). However, it is still not shown that p43 may be employed for treating wound. We identified that p43 has a novel activity of promoting wound healing process by acting on the wound healing process comprising the inflammation stage, epithelization stage and angiogenesis stage, and completed the present invention.

SUMMARY OF THE INVENTION

Therefore, it is an object of the present invention to provide a method for stimulating wound healing in a subject in need thereof, comprising administering to a wound of the subject an effective amount for stimulating wound healing of a composition, wherein the composition comprises p43 having an amino acid sequence set forth in SEQ ID NO: ~~1 or functional equivalents thereof~~

BRIEF DESCRIPTION OF THE DRAWINGS

The foregoing and other objects, features and advantages of the present

invention will become more apparent from the following detailed description when taken in conjunction with the accompanying drawings in which:

Fig. 1 is a schematic drawing showing where the gene trap is inserted within p43 mutant allele, wherein A, K, S and H indicate the restriction sites for Apa I , *Kpn* I , *Sac* I and *Hin*dIII, respectively. IF3, IR3 and pKOF2 are primers for PCR.

Fig. 2 shows the results of Southern blot (A) and PCR (B) for identifying the mutation of p43.

+/+: wild type mouse (WT);

+/-: heterozygous mutant mouse;

-/-: homozygous mutant mouse (Ho).

Fig. 3 shows the results of Northern blot (A) and Western blot for determining the expression of p43.

+/+: wild type mouse (WT);

+/-: heterozygous mutant mouse;

-/-: homozygous mutant mouse (Ho).

Fig. 4 is a photograph showing wound healing levels with the lapse of time (A) and a bar graph representing a percentage of wound healing levels to an initial wound area.

Ho: homozygous mutant mouse;

WT: wild type mouse which is not treated with p43 (control);

WT+p43: wild type mouse treated with p43.

Fig. 5 shows the result of histochemical analysis representing the effect of p43 to stimulate wound healing.

Arrows: advancing epithelial layers;

Purple region: granulation tissues.

Fig. 6 shows the results of immunostaining for determining the generation levels of macrophage/monocyte and endothelial cells.

Fig 7 is a bar graph representing the effect of p43 on the proliferation of fibroblasts.

Fig 8 shows the result of histochemical analysis representing the effect of p43 on the proliferation of fibroblasts.

Left: hematoxylin and eosin staining result of the crosssection of the wound area (arrows : the boundary of the wound area);

Right: immuno-fluorescence staining results of the boxed region in the wound area with the antibody against Ki-67.

Fig. 9 shows the results of wound migration assay (A), tube formation assay (B) and CAM assay (C) for estimating the effect of p43 in blood vessel generation.

Fig. 10 shows the results of the matrigel plug assay for confirming the stimulation of neovascularization by p43.

Fig. 11 is a bar graph representing the hemoglobin (Hb) contents within matrigel containing p43.

DETAILED DESCRIPTION OF THE INVENTION

According to an aspect of the present invention, there is provided a method for stimulating wound healing in a subject in need thereof, comprising administering to a wound of the subject an amount of a composition effective for stimulating wound healing, wherein the composition comprises a polypeptide selected from the group consisting of:

(a) a polypeptide having an amino acid sequence set forth in SEQ ID NO: 1; and

(b) a polypeptide having at least 70% sequence homology with the polypeptide of (a).

Hereinafter, the term "wound" includes any injury to any portion of the body of a subject including, but not limited to, acute conditions such as thermal burns, chemical burns, radiation burns, burns caused by excess exposure to ultraviolet radiation such as sunburn, damage to bodily tissues such as the perineum as a result of labor and childbirth, including injuries sustained during medical procedures such as episiotomies, trauma-induced injuries including cuts, incisions, excoriations, those injuries sustained in automobile and other mechanical accidents, and those caused by bullets, knives and other weapons, ulcer such as pressure ulcer, plaster ulcer and decubitus ulcer, and post-surgical injuries, as well as chronic conditions such as pressure sores, bedsores, conditions related to diabetes and poor circulation, and all types of acne. In addition, said wound includes dermatitis such as impetigo, intertrigo, folliculitis and eczema.

Areas of the body which can be treated with the present invention include, but are not limited to, skin, muscle and internal organs. Hereinafter, the term "subject" refers to a human or lower animal on whom the present invention is practiced.

The composition used in the present invention comprises, as an effective ingredient, p43 having an amino acid sequence set forth in SEQ ID NO: 1. The composition used in the present invention may also comprise the functional equivalents of the p43 as well as the p43. "Functional equivalents", as used herein, refer to polypeptides having the physiological activity to stimulate wound healing substantially equivalent with that of p43 having the amino acid sequences set forth in

SEQ ID NO: 1. The functional equivalents may be a polypeptide having at least 70% sequence homology, preferably at least 80%, more preferably at least 90%, and most preferably at least 95%, compared to the amino acid sequence set forth in SEQ ID NO: 1. The functional equivalents include amino acid sequence variants, for example, in which a portion or all of natural p43 amino acid sequence is substituted, or a portion of the p43 amino acid sequence is deleted or added. The substitution of the amino acid is preferably a conservative substitution. For example, the amino acids occurring the conservative substitution in nature include aliphatic amino acids (Gly, Ala, Pro); hydrophobic amino acids (Ile, Leu, Val), aromatic amino acids (Phe, Tyr, Trp), acidic amino acids (Asp, Glu), basic amino acids (His, Lys, Arg, Gln, Asn), and sulfur-containing amino acids (Cys, Met). The amino acid deletion is preferably located at a portion that does not affect the physiological activity of p43.

Herein, p43 or functional equivalents thereof can be administered (or applied) in an appropriate amount depending on the size of wound. In the case of a circular excision wound of bilateral symmetry having a diameter of 0.5cm, the p43 is preferably administered in a amount range of about 1ng to 5μg.

The composition comprising, as an effective component, the p43 or functional equivalents thereof according to the present invention may further comprise at least one selected from the group consisting of antibiotics, such as tetracycline, oxytetracycline, gentamicin, neomycin sulfate, bacitracin and polymyxin B sulfate; antihistamines, such as diphenhydramine, promethazine, tripelennamine, phenothiazine, chlorophenylamine, antazoline and pantholin; anti-inflammatory drugs; anti-viral drugs; anti-fungal agents; and growth factors, such as PDGF (platelet-derived growth factor), PDAF, PDEGF, TGF-β (transforming growth factor-β), PF-4, αFGF, bFGF (basic fibroblast growth factor), VEGF (vascular

endothelial growth factor), GH (growth hormone), EGF (epidermal growth factor) and IGF (insulin-like growth factor).

The composition used in the present invention may be compounded with a pharmaceutically acceptable carrier and employed in the form of powder, liniment, gel, lotion, cream, ointment, paste, puff, aerosol or suppository, and in particular, ointment and paste are more preferable. The carrier may comprise hydrocarbons including petrolatum, liquid paraffin and gelling hydrocarbon; animal or vegetable oils including medium chain triglyceride, pig fat, hard fat and cacao oil; higher fatty alcohols including cetanol and stearyl alcohol; fatty acids and esters thereof including stearic acid isopropyl palmitate; aqueous bases including polyethylene glycol, 1,3-butyleneglycol, glycerol, gelatin, white sugar and sugar alcohol; emulsifiers including glycerine fatty acid esters, polyoxyl stearate and polyoxyethylene hydrogenated castor oil; conglutinants including acrylate ester and sodium alginate; propellants including liquefied petroleum gas and carbon dioxide; and preservatives including paraoxybenzoates, depending on each formulation type.

When damaged skin was exposed to exterior environment, the damaged region may be infiltrated by toxins or bacteria, resulting in inflammation due to body's defense function. In treatment, the rapid generation of granulation tissue should be fostered in the damaged region and, above all, it is important for bacterial infection or excessive inflammation to be suppressed. Otherwise, not only much time is necessary in wound healing but also big scars will remain. The p43 having an amino acid sequence set forth in SEQ ID NO: 1 shows excellent effects on rapid healing of a wound by increasing granulation tissue and stimulating angiogenesis (blood vessel generation) in any wound including scald and ulcer regions.

The stimulation of wound healing by p43 identified in the present invention is considered to be a result from its multiple effect on inflammation as well as

angiogenesis. The attraction of macrophage/monocyte by p43 is consistent with our expectation because various proinflammatory cytokines and chemokines such as monocyte chemotactic protein 1 (MCP-1) or macrophage inflammatory protein 1α (MIP-1α) have been reported to be induced by p43 [Young-Gyu Ko, *et al.*, *J. Biol. Chem.* 276:23028-23033, 2001]. However, the pro-angiogenic activity of p43 shown in the present invention is surprising because p43 was previously shown to suppress the cell proliferation and induce apoptosis of endothelial cells at high concentration [Sun Young Chang, *et al.*, *J. Biol. Chem.*, 277:8388-8394, 2002]. Based on these results, the present invention provides the first evidences on a novel activity of p43 in angiogenesis associated with wound repair.

As can be seen from the foregoing, p43, an effective ingredient of the composition of the present invention has a novel physiological activity including the induction of macrophage/monocyte and endothelial cell, re-epithelization, proliferation of fibroblasts or angiogenesis. Therefore, it is expected that the method for stimulating wound healing using the composition containing the p43 or functional equivalents thereof according to the present invention can be extensively used in the general medical areas involving wound healing.

In a preferred embodiment of the present invention, the present inventors introduced a mutation to the structural gene of p43 in mouse. The mutation of the mouse genomic DNA may be effected according to conventional methods used in the prior art, including a gene trap method, a gene targeting method or an N-ethyl-N-nitrosourea (ENU) treatment method. Specifically, the gene trap vector was used herein to mutate the mouse genomic DNA. The gene trap vector may be one of those conventionally used in the prior art. More preferably, the gene trap vector

may be VICTR20 vector. The VICTR20 comprises two functional units, a neomycin resistant gene (*β-geo/neo^r*) and a puromycin resistant gene (*puro*) (see Fig. 1). The neomycin resistant gene is expressed by an upstream promoter of genomic DNA in which the vector is inserted and the puromycin resistant gene is expressed by the phosphoglycerate kinase-1 (PGK) promoter of the vector. As shown in Fig. 1, the gene trap vector, VICTR20 includes long terminal repeats (LTRs) in both ends, a splice accepter (SA), a neomycin resistant gene (*neo^r*), a poly-A sequence (pA), a PGK promoter, a puromycin resistant gene (*puro*) and a splice donor (SD).

Then, to construct a mutant library, the genomic DNA in which a mutation is derived from the insertion of the gene trap is introduced into embryonic stem cells of mice. In the mutant library, the clone containing the disrupted p43 gene is identified and used to prepare heterozygous mutant mice.

In another preferred embodiment of the present invention, the nucleotide sequence of p43 allele in the p43 mutant mice is determined, and thereby it can be confirmed that the gene trap vector is inserted at the first intron of the p43 gene (See Fig. 1). The mutation of p43 is determined by Southern blot and PCR analysis (See Fig. 2) and the mutational effect on the expression of p43 is determined by Northern and Western blot (See Fig. 3). The mating of the heterozygous mutant mice generated 16 of wild type, 53 of heterozygous mutant mice and 28 of homozygous mutant mice. This ratio is close to the Mendelian segregation, indicating no significant embryonic lethality of the mutant mice. Interestingly, the homozygous mutants significantly reduced body size compared to the wild type mice (data not shown).

It has been reported that p43 is associated with immune mechanism and works on endothelial cells [Chang S.Y., *et al.*, *J. Biol. Chem.*, 277:8388-8394, 2002; Park H-Y., *et al.*, *J. Leukocyte Biol.* 71:223-230, 2002; Ko Y-G., *et al.*, *J. Biol.*

Chem. 276:23028-23033, 2001]. We expected that p43 might be involved in the processes of inflammation and angiogenesis occurring in the wound healing process. Thus, to confirm our expectation, the biopsies were introduced to the wild type and homozygous mutant mice, and the healing process was compared by gross examination and histological inspection. The wounds in the mutant mice (Ho) recovered at much slower rate compared to those in the wild type mice and direct administration of the purified p43 to the wound region of the wild type mice stimulated the healing process (See Fig. 4). The cross-sections of the wound showed the increase of granulation tissue and rapid re-epithelization in the wounds of the wild type mice treated with p43 (See Fig. 5).

Further, immunostaining of the wound regions with an antibody that is specific to macrophage/monocyte showed the enrichment of these cells in the wound region tissue of the p43-treated wild type mice (WT+p43) while fewer numbers of these cells were observed in the wound region tissue of the p43 mutant mice (Ho) (See Fig. 6). The macrophage/monocyte-specific antibody may include, but not limited to, anti-MOMA-2 antibody. The immunostaining of the wound regions with an antibody specific to endothelial cells showed that vascularization was also significantly enhanced in the p43-treated wild type mice (WT+p43) while the reverse was the case in the p43 mutants (Ho) (See Fig. 6). The antibody specific to endothelial cell may include, but not limited to, anti-CD31 antibody.

To determine the effect of p43 on the proliferation of different cells in the wound region, we have treated the cultivated foreskin fibroblast cells with p43. The proliferation of fibroblasts was significantly increased in dose-dependent manner by p43 (See Fig. 7). p43 did not stimulate the proliferation of keratinocyte and endothelial cells (data not shown). To see whether p43 can also show the proliferative effect on the fibroblasts in the dermal region of the wound area, we have

generated the wound in the back skin of the mice and treated p43 after wounding twice a day in every other day. The proliferation of dermal fibroblasts was then compared by the immuno-fluorescence staining with anti-Ki67 antibody 3 days after wounding. The proliferation of dermal fibroblasts was stimulated in the p43 treated wound region (See Fig. 8). This result is consistent with the effect of p43 on the cultivated fibroblasts. All of these results indicate that p43 can stimulate the proliferation of fibroblasts.

To further clarify that p43 stimulates the generation of endothelial cell and induces angiogenesis, the present inventors carried out several different experiments that can determine the activity of p43 in angiogenesis.

First, the present inventors investigated whether p43 induces the migration of endothelial cells by cellular wound migration assay. In this assay, the endothelial cell migration was enhanced in the presence of p43 (See Fig. 9A). Then, the endothelial cells were cultivated on Matrigel and then the stimulated tube formation was also observed in the presence of p43 (See Fig. 9B). Secondly, chorioallantoic membrane assay (CAM) was conducted. It could be seen that blood vessels were attracted to the area to which p43 was spotted (See Fig. 9C).

Lastly, the matrigel plugs containing p43 were used to see how p43 affects neovascularization. As a positive control, the present inventors used basic fibroblast growth factor (bFGF) which was known to be associated with heparin in various forms and then to promote angiogenesis, epithelization and the deposition of collagen fiber (Tsuboi, R. *et al.*, *J. Exp. Med.* 172:245, 1990; Kinsnorth, A. N. *et al.*, *Br. J. Surg.* 77:409, 1990). The blood vessel formation within the gel was monitored by immunostaining with the antibody specific to endothelial cells. It could be confirmed that the gels containing bFGF or p43 showed strongly enhanced

neovascularization (See Fig. 10). The result of immunostaining with the antibody specific to macrophage/monocyte was consistent with the result above (See Fig. 10). Further, it could be seen that the gels containing bFGF or p43 contained higher concentration of red blood cells than the control (See Figs. 10 and 11).

BEST MODE FOR CARRYING OUT THE INVENTION

This invention is further illustrated by the following examples, which are not be construed in any way as imposing limitations upon the scope thereof.

Example 1: Preparation of p43 mutant mouse

To prepare p43 mutant mice, p43 structural gene was allowed to be mutated using the gene trap method [Cecconi, F. & Meyer, B. I., *FEBS Lett.*, 480:63-71, 2000]. The gene trap vector, VICTR20 (Lexicon Genetics, USA) was used to mutate the genomic DNA of SvEvBrd mice (Lexicon Genetics, USA) according to Zambrowicz's method [Zambrowicz, B. P. *et al.*, *Nature* 392:608-611, 1998]. The mutated genomic DNA was introduced into the embryonic stem cells derived from 129/SvEvBrd mice (Omnibank) to generate the mutant library. In the library, the clone containing p43 gene disrupted by the insertion of the gene trap vector was screened and called 'OST58507'. The clone was used to generate the heterozygous C57/BL6 mice (Samtako) by the standard method of Lexicon Genetics. The mating of the heterozygous mice generated 16 of wild type, 53 of heterozygous mutant mice and 28 of homozygous mutant mice. The ratio is close to the Mendelian segregation, indicating no significant embryonic lethality of the mutant.

Example 2: Characterization of p43 mutant mouse

2.1 Determination of the gene trap vector's site inserted into p43 allele

To determine the site where the gene trap vector was inserted into p43 allele, the p43 mutant allele was analyzed by sequencing. The sequencing was determined by Pangenomcis, a company for sequence analysis. As shown in Fig. 1, the first exon (Exon I) and second exon (Exon II) of p43 mutant gene were separated by the intron of about 7kb. It was confirmed that the gene trap vector was inserted at about 1.5kb downstream of the first exon.

2.2 Southern blot

The genomic DNA was isolated from the tail of each mouse and digested with *Nco*I. The cleaved DNA fragments were separated by denaturing gel electrophoresis. Then, southern blot analysis was performed using the neomycin resistant gene (*Neo*^{r})(SEQ ID NO: 2) of the gene trap vector or the *Hind*III fragment (SEQ ID NO: 3) of the p43 mutant gene as a prove, according to a conventional method [Southern, E. M., *J. Mol. Biol.*, 98:503, 1975]. As shown in Fig. 2A, the *Neor* gene of the gene trap vector and the p43 mutant gene (7kb) were detected in the homozygous mutant mice. However, the *Neo*^{r} gene was not detected in the wild type mice. In the heterozygous mutant mice, the *Neo*^{r} gene was mildly detected and both intact p43 gene (10kb) and the p43 mutant gene (7kb) were detected. The size of the p43 mutant gene (7kb) was smaller than that of the intact p43 gene (10kb) because of the presence of *Nco* I site within *Neo*^{r} gene of the gene trap vector. From the results, it can be demonstrated that the p43 mutant mouse of the present invention contains a mutation within the p43 gene.

2.3 PCR

To amplify the 1kb DNA fragment containing the first intron of the p43 gene, the genomic DNA was used as a template for PCR analysis with IF3 primer (SEQ ID NO: 4) and IR3 primer (SEQ ID NO: 5) (See Fig. 1). To amplify the 2.2kb DNA fragment containing a portion of the gene trap vector and a portion of the p43 gene, pKOF2 primer (SEQ ID NO: 6) and the IR3 primer were used in PCR analysis (See Fig. 1). For the PCR analysis, the template DNA was denatured at a temperature of 94℃ for 5 min and cycled 25 times at 94℃ for 1 min, at 57℃ for 1min and at 72℃ for 1min. As can be seen from Fig. 2B, only one band of 1kb was detected in the wild type mice (+/+) and two bands corresponding to 1kb and 2.2kb, respectively, were detected in the heterozygous mutant mice (+/-). In the homozygous mutant mice (-/-), a band of 2.2kb was only detected. From the results, it can be demonstrated that the p43 mutant mouse of the present invention contains a mutation within the p43 gene.

Example 3: Expression of p43 by p43 mutant mouse

3.1 Northern blot

For Northern analysis, total cellular RNA was prepared from mouse embryonic fibroblast (MEF) cells using RNeasy Midi Kit (QIAGEN) according to the manufacturer's instruction. The isolated RNA was separated on denaturing gel, and then transferred to the Hybond-N^+ membrane (Amersham). The RNAs on the membranes were hybridized with the specific probes for p43 set forth in SEQ ID NO: 7. As shown in Fig. 3A, p43 mRNA was detected in the wild type (+/+) and the heterozygous mutant mice, but was not detected in the homozygous mutant mice (-/-). It indicates that p43 is not expressed in p43 mutant mice (-/-) of the present

invention.

3.2 Western blot

For Western blot analysis, the protein was isolated from the mouse organ according to Ziak *et al.* (Ziak M, *et al.*, *Biochem. Biophys. Res. Commun.* 280:363-367, 2001). Western blotting was carried out with anti-p43 antibody as described previously [Park S. G., *et al.*, *J. Biol. Chem.* 274:16673-16676, 1999]. As shown in Fig. 3B, p43 protein was detected in the wild type (+/+) and the heterozygous mutant mice, but was not detected in the homozygous mutant mice (-/-). It was consistent with the result of the Northern blot.

Example 4: Stimulatory activity of p43 in wound healing

4.1 Gross appearance

For gross appearance, the back skin and *panniculus carnosus* muscle of 7 to 8 weeks old mice (wild type and homozygous mutant mice) were excised according to Werner *et al.*[Werner, S., *et al.*, *Science* 266:819-822, 1994]. Firstly, the present inventor disinfected the back skin of mice with 70% ethanol, and shaved fur from the back under avertin anesthetization. Then, the circular excision wound of bilateral symmetry having a diameter of 0.5 cm was prepared using a circular punch of 1 cm on the back skin. The wounds were left uncovered without the drug treatment or dressing. Meanwhile, p43 was prepared from *E.coli* as described previously [Park S. G., *et al.*, *J. Biol. Chem.* 274:16673-16676, 1999]. 4μg of the purified p43 was dissolved in 5$\mu\ell$ of PBS buffer containing 20% glycerol. Each group of the wounds in 25 wild type mice was treated with either p43 (4ug/wound) or the PBS buffer with 20% glycerol alone (control), 4 times at 2 days interval. Also, the p43

mutant mice were left without the treatment of p43. The wound closure was monitored daily using the Image-Pro Plus Software (Media Cybernetics).

As shown in Fig. 4A, the wounds in the wild type mice treated with p43 (WT+p43) recovered at much faster rate compared to those in the homozygous mutant mice (Ho) or the wild type mice which were not treated with p43 with the lapse of time. However, the wounds in the homozygous mutant mice (Ho) recovered at a much slower rate compared to those in the wild type mice which were not treated with p43 even after much time had passed. The wound closure was calculated as the percentage of the initial wound area. As shown in Fig. 4B, the wound area of the wild type mice treated with p43 (WT+p43) was significantly decreased with the lapse of time.

4.2 Histochemical analysis

5 day-aged wounds were isolated with 3mm of adjacent normal tissue, fixed overnight in Bouin's solution (Sigma, HT10-1-32), and then embedded in paraffin. Paraformaldehyde-fixed paraffin sections (6μm) of the wounds were stained by the Masson Trichome procedure (Lund, L. R., *et al.*, *EMBO J.* 18:4645-4656, 1999). Only littermates of the same sex were used for direct histological comparison.

As shown in Fig. 5, the wild mice treated with p43 (WT+p43) were confirmed to indicate the increase of granulation tissue (purple sites below the wound areas) and rapid re-epithelialization by the increase of advancing epithelial layer (arrows). However, the homozygous mutant mice (Ho) showed little increase of advancing epithelial layer in the wounds.

4.3 Induction of macrophage/monocyte and endothelial cells by p43

5 day-aged wounds were isolated with 3mm of adjacent normal tissue.

The isolated wounds were fixed in 4% paraformaldehyde in PBS and then frozen in the OCT compound (Sakura). The cyro-sections of the wounds were analyzed by immune-fluorescence. To detect macrophage/monocyte and endothelial cells, the frozen sections were incubated in 1.7μg/ml of anti-mouse MOMA-2 antibody (Serotec) and 1.0μg/ml of anti-CD31 antibody (Pharmingen) that are specific to the cells, respectively. After incubation for 1 hour with the primary antibodies, 1.0 μg/ml of FITC-conjugated secondary antibody was added and further incubated for 1 hour. The wounds were then counterstained with propidium iodide (1μg/ml), mounted on slides and investigated using confocal immunofluorescence microscopy (μRadience; BioRad).

As shown in Fig. 6, the wound tissues of the wild type mice treated with p43 (WT+p43) showed the enrichment of the macrophage/monocyte while the wound tissues of the homozygous mutant mice (Ho) showed fewer numbers of the macrophage/monocyte. Also, the immunostaining of the wounds with the antibody specific to endothelial cells showed that vascularization was also significantly enhanced in the p43-treated wounds while the reverse was the case in the p43 mutants (See Fig. 6).

4.4 Effect of p43 on the proliferation of fibroblasts

4.4.1 Effect of p43 on the cultivated fibroblasts

To determine the effect of p43 on the proliferation of foreskin fibroblasts, the foreskin fibroblasts(American Type Culture Collection) were cultivated in the presence of the different amounts of p43 (1, 10, and 100 ng/ml) for 48 h, and then in fresh medium conaining 1 uCi tritium-labeled thymidine for 4 h. After washing and lysing the cells with RIPA buffer, the amounts of the incorporated thymidine were

quantified by liquid scintillation counting.

As shown in Fig.7, the proliferation of fibroblasts was significantly increased in dose-dependent manner by p43.

4.4.2 Histological analysis

To determine the effect of p43 on the proliferation of dermal fibroblasts in the cutaneous wound area, the cutaneous wounds were generated on the back skin of 7 to 8 weeks old mice mice according to Werner *et al.*[Werner, S., *et al.*, *Science* 266:819-822, 1994]. Meanwhile, p43 was prepared from *E.coli* as described previously [Park S. G., *et al.*, *J. Biol. Chem.* 274:16673-16676, 1999]. 4μg of the purified p43 was dissolved in 5$\mu\ell$ of PBS buffer containing 20% glycerol. The wounds in 25 wild type mice were treated with either p43 (4ug/wound) or the PBS buffer with 20% glycerol alone (control), twice in every other days. The mice were sacrificed in 3 days after wounding and the wound area was isolated for histological analysis. The crosssection of the wound area was stained using hematoxylin and eosin. The boxed region in the wound (see Fig 8 Left) was analyzed by mmuno-fluorescence with the antibody against Ki-67 that is the marker of the cell proliferation (Ben-Izhak, O. et al. Ki67 antigen and PCNA proliferation markers preducts survival in anorectal malignant melanoma. Histopathology 41, 519-525, 2002). 3 day-aged wounds were isolated with 3mm of adjacent normal tissue. The isolated wounds were fixed in 4% paraformaldehyde in PBS and frozen in OCT compound. The frozen sections (6 μm) were equilibrated 3 times with PBS for 5 min. Non- specific interactions were blocked with PBS containing 0.1% Tween 20, 1% non fat milk for 2 h at room temperature and sections were incubated with 1/50 diluted anti-KI67 antibody (Santacruz) in PBST(PBS containing 0.1% Tween 20) for 2 hrs at 37°C. The sections were washed 2 times with PBST and subsequently

incubated for 1 h at 37°C with the FITC conjugated antibody. The sections were counterstained with propium iodide (10ug/ml) for 15 min and washed 3 times with PBST for 5 min. Fluorescence signals were examined under the confocal immunofluorescence microscopy (Radiance, BioRad).

As shown in Fig.8, p43 can stimulated the proliferation of fibroblasts.

Example 5: Effect of p43 on angiogenesis

5.1 Cellular wound migration assay

To investigate whether p43 induces the migration of endothelial cells, the cellular wound migration assay was performed. First, aorta was excised from the butchered cattle and washed by dipping in cold PBS buffer containing 1% penicillin and 0.25μg/ml fungizone. Then, bovine aortic endothelial cells (BAECs) were isolated from the surface of the tracheal aorta using a razor blade and incubated in DMEM containing 20% fetal bovine serum (FBS). The incubated cell cultures were wounded with a razor blade in 2 mm in width and the injury lines were marked. Then, the cultures were washed with serum-free medium and further incubated in DMEM with 1% serum, 1mM thymidine and 1nM p43. The cells were allowed to migrate for 16 hours and then rinsed. A control was not treated with p43. The cultures were fixed with absolute methanol (100%) and stained with Giemsa (Chameleon Chemical).

As shown in Fig. 9A, the endothelial cell migration was enhanced in the presence of p43. From the result, it was demonstrated that p43 stimulates the endothelial cell migration.

5.2 Blood tube formation

From the result of Example 5.1 described above, it was confirmed that p43 induced the endothelial cell migration. And then, to investigate whether p43 induces blood tube formation, BAECs ($5x10^5$ cells) were cultivated on Matrigel (Becton Dickinson) in the presence of 1nM of p43 at 37℃ for 6 hours. A control was not treated with p43. Then, the changes of cell morphology were captured by phase contrast microscopy.

As shown in Fig. 9B, the stimulated tube formation was observed in the presence of p43, but in the control. The result indicated that p43 stimulates tube formation and it was consistent with the endothelial cell migration.

5.3 Chorioallantoic membrane assay

Fertilized chick eggs were incubated in the humidified egg breeder at 37℃. On the third day of incubation, about 2ml of egg albumin was removed by an 18-gauge hypodermic needle to detach the developing chorioallantoic membrane (CAM) from the shell. After the incubation for additional 6 days, thermanox coverslips (Nunc) loaded with 0.1μg of p43 (dissolved in PBS buffer containing 20% glycerol) were placed on the CAM surface and vascularization was observed. A control was not treated with p43. As shown in Fig. 9C, blood vessels were attracted to the area to which p43 was spotted.

5.4 Matrigel plug assay

To see how p43 affects neovascularization, matrigel plugs containing p43 were subcutaneously injected into the skin of mice and stained by Masson Trichome method, and then red blood cells within the gels were counted. First, the Matrigel (0.25ml, Becton Dickinson) was supplemented with p43 (1ng/ml) plus heparin (9U/ml, Sigma). PBS buffer containing 20% glycerol was used as a negative

control. And as a positive control, 143 ng/ml of bFGF (R&D systems) plus heparin was used, in which bFGF was known as a growth factor to stimulate the blood tube formation by associating with heparin in a various form. The mixtures were subcutaneously injected into 7 weeks old C57BL6/J mice (Samtako) in a dose of 0.25 ml/site. The mice were sacrificed 7 days after the inoculation and the gels were removed, fixed in 4% paraformaldehyde for 24 hours. The gel plugs were embedded in the OCT compound (Sakura) and then sliced into sections of 10 μm thick using Cyo-microtome (Zeiss). The sections were reacted with anti-CD31 antibodies (Pharmingen) and anti-MOMA2 antibodies (Serotec) that are specific to the endothelial cells and macrophage/monocyte, respectively, and then stained by Masson Trichome method [Lund, L. R., *et al.*, *EMBO J.* 18:4645-4656, 1999]. As a result, in the presence of p43, neovascularization was strongly enhanced (See Fig. 10, top row) and macrophage/monocyte were also recruited to the gel compared to the control (See Fig. 10, middle row, green fluorescence). Interestingly, other cells were also enriched in the gels treated with p43 (red fluorescence).

The blood vessel formation within the gels was also monitored by counting red blood cells. As shown in Fig. 10 (bottom row, arrowheads), the gels containing bFGF or p43 contained higher concentration of red blood cells than the control. Further, the concentration of hemoglobin within the gels was determined as described previously [Passaniti, A., *et al.*, *Lab. Invest.*, 67:519-528,1992]. As shown in Fig. 11, the gels containing bFGF or p43 contained higher concentration of hemoglobin than the control.

While this invention has been described in connection with what is presently considered to be the most practical and preferred embodiment, it is to be understood that the invention is not limited to the disclosed embodiment and the drawings, but,

on the contrary, it is intended to cover various modifications and variations within the spirit and scope of the appended claims.

The entire disclosure of Korea Patent Application No. 2002-42858, filed on July 22, 2002 including its specification, claims, drawings and summary are incorporated herein by reference in its entirety.